INFECTION AND IMMUNITY, Oct. 1995, p. 3796–3803
0019-9567/95/$04.00+0

Vol. 63, No. 10

Molecular Cloning of the cDNA and Gene for an Elastinolytic Aspartic Proteinase from *Aspergillus fumigatus* and Evidence of Its Secretion by the Fungus during Invasion of the Host Lung

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Received 21 December 1994/Returned for modification 7 February 1995/Accepted 10 July 1995

Hydrolysis of structural proteins in the lung by extracellular proteinases secreted by *Aspergillus fumigatus* is thought to play a significant role in invasive aspergillosis. This fungus was found previously to secrete an elastinolytic serine proteinase and a metalloproteinase. We report that *A. fumigatus* also secretes an aspartic proteinase (aspergillopepsin F) that can catalyze hydrolysis of the major structural proteins of basement membrane, elastin, collagen, and laminin. The pH optimum for the enzymatic activity was 5.0 with elastin-Congo red as the substrate, and the activity was not significantly inhibited by pepstatin A, diazoacetyl norleucine methylester, and 1,2-epoxy-3-(*p*-nitrophenoxy) propane. The cDNA and gene encoding this aspartic proteinase were cloned and sequenced. The open reading frame, interrupted by three introns, would encode a protein of 393 amino acids composed of a putative 21-amino-acid signal peptide and a 49-amino-acid propeptide preceding the 323-amino-acid mature protein. The amino acid sequence of *A. fumigatus* aspartic proteinase has 70, 66, and 67% homology to the sequences of those from *Aspergillus oryzae*, *Aspergillus awamori*, and *Aspergillus saitoi*, respectively. The active-site motif (DTG) and the catalytic aspartic residues characteristic of aspartic proteinases are found in the presently described enzyme, indicating that it belongs to a family of aspartic proteinases. Polyclonal antibodies were produced in rabbits against both the mature and precursor forms of the aspartic proteinase expressed in *Escherichia coli*. Immunoblotting with both antibodies detected a 39-kDa mature enzyme in the culture supernatant of *A. fumigatus*. The aspartic proteinase activity was inhibited by the antibodies, suggesting that the aspartic proteinase in the culture supernatant corresponds to the product of the cloned gene. Immunogold electron microscopy showed that the aspartic proteinase was secreted by *A. fumigatus* invading neutropenic mouse lung and its secretion was directed toward the germ tubes of penetrating hyphae.

Invasive aspergillosis is a major threat to the long-term survival of immunocompromised patients, such as bone marrow transplant patients (6). Even with the best antifungal agents, such as amphotericin B, the mortality rate ranges from 50 to 95% (1, 5). Approaches targeted at the virulence factors might provide new methods for prevention and treatment of the disease. Extracellular proteinases which can hydrolyze the structural components of the lung are thought to be possible virulence factors. Since elastin constitutes 30% of the lung (39), elastinolytic enzymes are thought to play a significant role in the fungal penetration. Production of elastinolytic serine proteinases has been reported for both *Aspergillus fumigatus* and *Aspergillus flavus*, two major causative agents of invasive aspergillosis (13, 28). Evidence that the elastinolytic proteinases from *A. fumigatus* constitute significant virulence factors has been presented (13). Alkaline serine proteinase gene-disrupted mutants of *A. fumigatus* were reported to retain virulence in murine models (22, 40). However, the mutants were found to produce a metalloproteinase (21). Extracellular elastinolytic metalloproteinases produced by *A. fumigatus* (19) and *A. flavus* (30) have been isolated and characterized, and the metalloproteinase was shown to be secreted during the invasion of lungs of neutropenic mice by *A. fumigatus* (19). Thus, metalloproteinase might compensate for the lack of the serine proteinase in the gene-disrupted mutants. Aspartic proteinases from *Aspergillus* species have not been examined as possible virulence factors in invasive aspergillosis, although such enzymes are known to be virulence factors in candidiasis (14). In this report, we present evidence that *A. fumigatus*

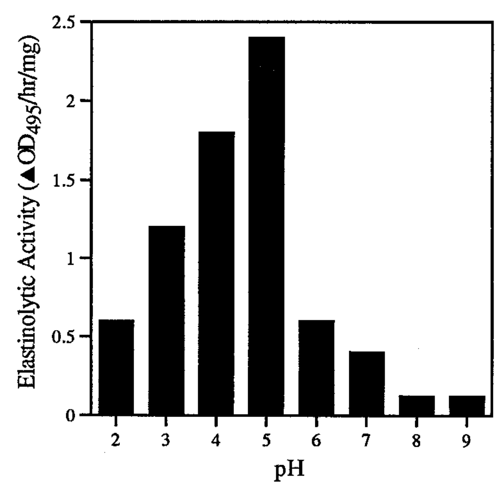

FIG. 1. Effect of pH on *A. fumigatus* aspartic proteinase activity. The culture supernatant was preincubated with PMSF (2 mM) and 1,10-phenanthroline (2 mM) in the buffer for 20 min at room temperature before the assay with elastin-Congo red as described in the text. Buffers used were 50 mM sodium acetate for pH 2.0 to 5.0 and 50 mM Tris-HCl for pH 6.0 to 9.0. OD_{495}, optical density at 495 nm.

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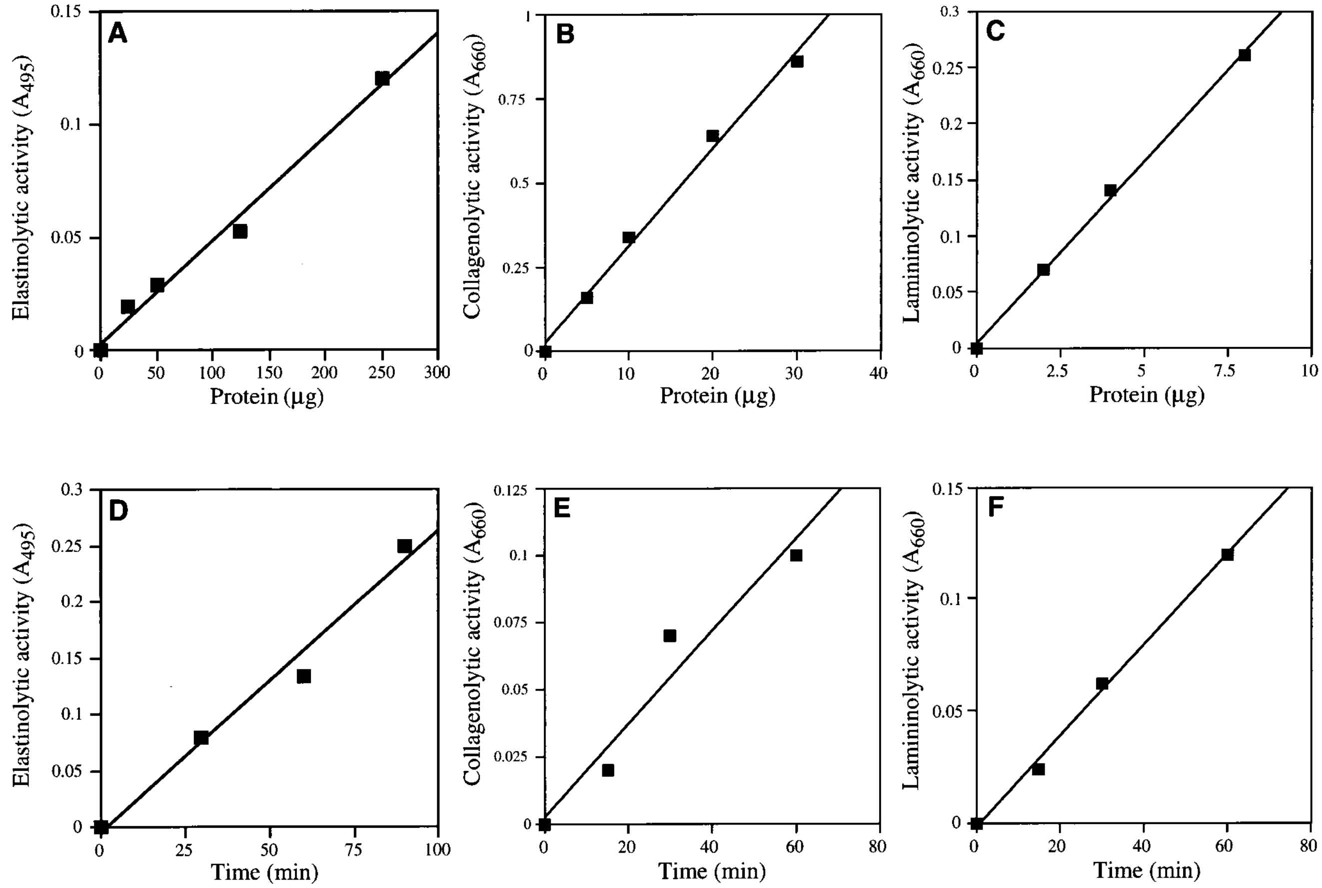

FIG. 2. Elastinolytic (A and D), collagenolytic (B and E), and lamininolytic (C and F) activities of the aspartic proteinase of *A. fumigatus*. The culture supernatant was preincubated with PMSF (2 mM) and 1,10-phenanthroline (2 mM) for 20 min before starting the assay, which was done in 0.1 M sodium citrate buffer (pH 4.2) for 1 h (A to C) or the indicated periods (D to F) at 37°C with elastin-Congo red, type I collagen, or laminin as the substrate.

produces an aspartic proteinase that is capable of hydrolyzing the major structural barriers of the lung and present immunoelectron microscopic evidence that such a proteinase is secreted during the fungal invasion into the lungs of neutropenic mice. The molecular cloning of the cDNA and gene for the enzyme and a comparison of its primary structure with other known fungal aspartic proteinases (aspergillopepsins) are also presented.

MATERIALS AND METHODS

Microorganisms and culture conditions. *A. fumigatus* isolate 13 used in this study and the culture conditions for *A. fumigatus* were described elsewhere (13). *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host for recombinant plasmids, and cells were grown at 37°C in Luria broth medium. *E. coli* Y1090 r^- was used as the host for bacteriophage λ. pBluescript KS^+ was used to subclone various DNA fragments and to prepare double-stranded DNA for sequencing.

Proteinase assays. The proteinase activity with *N′,N′*-dimethyl hemoglobin (Sigma Chemical Co., St. Louis, Mo.) was measured in 0.1 M sodium acetate buffer at pH 4.2 at 37°C for 30 min as described by Lin et al. (16). Elastinolytic activity with ^{3}H-elastin as the substrate was measured as described previously (13). Elastinolytic activity with elastin-Congo red (Sigma) was determined in 50 mM sodium acetate (pH 5.0) in a 1-ml volume containing 5 mg of elastin-Congo red (37). After 1 h of incubation, the insoluble materials were removed by centrifugation at 12,000 × *g* for 10 min, and the pH of the supernatant was raised to 7.0 by the addition of an equal volume of 1 M Tris buffer (pH 7.0), before the measurement of A_{495}. Collagenolytic activity was determined in a 1-ml volume containing 2 mg of type I collagen (Sigma). After the indicated period of time, the proteins in the reaction mixture were precipitated with trichloroacetic acid at a final concentration of 5% and removed by centrifugation. The supernatant was

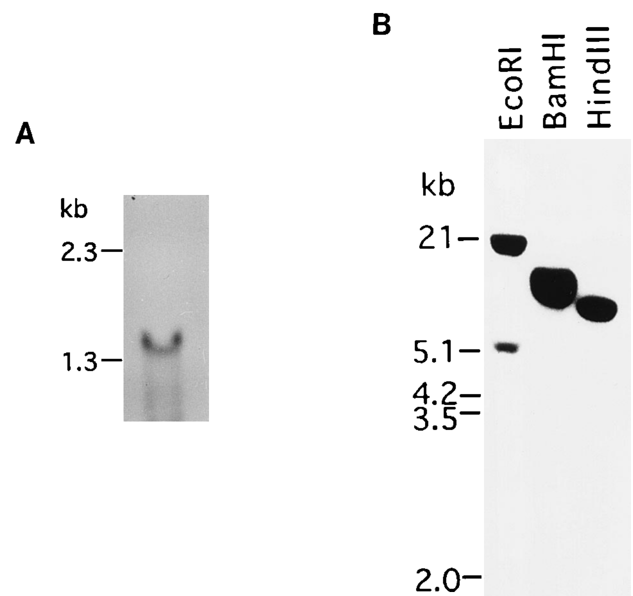

FIG. 3. (A) Northern hybridization of total RNA of elastin-grown *A. fumigatus* with the 600-bp PCR product corresponding to a segment of the aspartic proteinase gene as the probe; (B) Southern hybridization of *A. fumigatus* genomic DNA digested with the indicated restriction enzymes with the entire cDNA of the aspartic proteinase as the probe.

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   1 tacatgtccctggatattgtagatcttcatccggactacctggataagagatatggcgtgaaggcataggtgaaacttgc 80
  81 tcctcctggagccggagtgattccgatagtaacttctatctctcctgggcccatggacaaggggccccactaaatatacta 160
 161 tataaaggaggcatgtttcaccagctgttgaccgaatatcatcctcattctcgttcagcaagagtttgagatcgtctcct 240
 241 tttttgtcgttgatacttttccacggtccagtagaccgttgtttcatcaag ATG GTC GTC TTT AGC AAA GTC  312
   1                                                    M   V   V   F   S   K   V    7
 313 ACC GCT GTC GTC GTC GGT CTC TCG ACC ATT GTG ATG CTG TCC CTG TGG TCC AGC CGC GCC  372
   8 T   A   V   V   V   G   L   S   T   I   V   M   L   S   L   W   S   S   R   A    27
 373 AAG GGC TTC ACT ATC TTC CTT GTG GCC AGA CCA GTG ACC AAC AAG AAG ACC GTC AAT CTT  432
  28 K   G   F   T   I   F   L   V   A   R   P   V   T   N   K   K   T   V   N   L    47
 433 CCA GCT GTC TAT GCC AAT GCT TTG ACT AAG TAC GGG GGC ACT GTC CCC GAC AGT GTC AAG  492
  48 P   A   V   Y   A   N   A   L   T   K   Y   G   G   T   V   P   D   S   V   K    67
 493 GCG GCT GCA AGC TCC GGC AGC GCT GTT ACT ACC CCC GAG CAA TAT GAC TCG GAA TAC CTG  552
  68 A   A   A   S†  S   G   S   A   V   T   T   P   E   Q   Y   D   S   E   Y   L    87
 553 ACC CCC GTC AAA GTC GGT GGA ACG ACC CTG AAC TTG GAC TTC GAC ACT GGC TCT GCA GAT  612
  88 T   P   V   K   V   G   G   T   T   L   N   L   D   F   D   T   G   S   A   D    107
                                                          * *** *** *** *** *** ***
 613 CT gtaagcgcatcttgtccctctttcttgaattgaattcactaattgttgtag C TGG GTC TTC TCC TCC GAG 684
 108 L                                                         W   V   F   S   S   E    114
 685 CTT TCG GCT TCC CAG TCC AGC GGC CAT GCT ATC TAC AAG CCG TCC GCT AAT GCC CAA AAG  744
 115 L   S   A   S   Q   S   S   G   H   A   I   Y   K   P   S   A   N   A   Q   K    134
 745 CTG AAT GGC TAC ACC TGG AAG ATC CAA TAT GGT GAT GGT AGC AGT GCC AGC GGT GAC GGC  804
 135 L   N   G   Y   T   W   K   I   Q   Y   G   D   G   S   S   A   S   G   D   G    154
 805 TAC AAG GAT ACC GTC ACT GTG GGT GGT GTC ACT GCT CAG AGC CAG GCT GTG GAG GCT GCC  864
 155 Y   K   D   T   V   T   V   G   G   V   T   A   Q   S   Q   A   V   E   A   A    174
 865 AGC CAT ATC AGC TCT CAA TTC GTG CAG GAT AAG GAC AAC GAT GGT CTG TTG GGT TTG GCA  924
 175 S   H   I   S   S   Q   F   V   Q   D   K   D   N   D   G   L   L   G   L   A    194
 925 TTC AGC TCC ATC AAC ACT G gtgagtttcagtcaagattaccagcttcacctcagcccactgactcggattgtt 997
 195 F   S   S   I   N   T   V                                                          201
 998 acag TC AGT CCC TCA GAC TAC TTT CTT TAC ACT GTC AAG TCC CAG TTG GAC TCT CCT CTC  1057
 202         S   P   S   D   Y   F   L   Y   T   V   K   S   Q   L   D   S   P   L    219
1058 TTT GCT GTG ACC TTG AAG TAC CAT GCT CCA GGC ACC TAC GAC TTT GGA TAC ATC GAC AAC  1117
 220 F   A   V   T   L   K   Y   H   A   P   G   T   Y   D   F   G   Y   I   D   N    239
1118 TCC AAG TTC CAA GGG GAA CTC ACT TAT ACC GAC GTC GAC AGC TCC CAG GGT TTC TGG ATG  1177
 240 S   K   F   Q   G   E   L   T   Y   T   D   V   D   S   S   Q   G   F   W   M    259
1178 TTC ACT GCT GAT GGC TAC GGT GTT GGC AAT GGT GCT CCC AAC ACC AAC AGT ATC AGC GGC  1237
 260 F   T   A   D   G   Y   G   V   G   N   G   A   P   N   T   N   S   I   S   G    279
                                                                ** *** *** *** ***
1238 ATT GCT G gtatgttttcatagtgtctttcagaaggaccaagctgatccacttttag AC ACC GGC ACC ACC   1307
 280 I   A   D                                                      T   G   T   T     286
     *** ***
1308 CTC CTC CTG CTT GAT GAC AGC GTT GTT GCC GAC TAC TAC CGC CAG GTT TCC GGA GCC AAG  1367
 287 L   L   L   L   D   D   S   V   V   A   D   Y   Y   R   Q   V   S   G   A   K    306
1368 AAC AGC AAC CAA TAC GGT GGT TAT GTC TTC CCC TGC TCC ACC AAA CTT CCT TCT TTC ACT  1427
 307 N   S   N   Q   Y   G   G   Y   V   F   P   C   S   T   K   L   P   S   F   T    326
1428 ACC GTC ATC GGA GGC TAC AAT GCC GTC GTT CCC GGT GAA TAC ATC AAC TAC GCC CCG TCA  1487
 327 T   V   I   G   G   Y   N   A   V   V   P   G   E   Y   I   N   Y   A   P   S    346
1488 CTG ACG CAG CTC TAC CTG CTA CGG CGG CAT CAG AGC AAC TCT GGT CTG GGC TTT TCT ATC  1547
 347 L   T   Q   L   Y   L   L   R   R   H   Q   S   N   S   G   L   G   F   S   I    366
1548 TTC GGA GAT ATC TTC CTC AAG AGC CAG TAC GTC GTC TTC GAC TCC CAA GGC CCC AGA CTC  1607
 367 F   G   D   I   F   L   K   S   Q   Y   V   V   F   D   S   Q   G   P   R   L    386
1608 GGC TTC GCC CTC AGG CAT AGA TGA atatgcacgcggttttggtctttgaggccggcctggccgatctatgg 1679
 387 G   F   A   L   R   H   R                                                          394
1680 ggtctgtgaccggcgagaatcatctccagtttgttcttgtatatggttttagtttagaatagcaatatgattatgacact 1759
1760 gaggcctttgtacgtgcaatagcatgatagcaactatgcgatcagagatccagacaccctgtttcgacaaagattttggc 1839
1840 agcgccgcatattcacggatgagtagtttcgaacacgccaatgg                                     1883
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FIG. 4. Nucleotide sequence of *A. fumigatus* gene encoding aspartic proteinase and the deduced amino acid sequence. The putative TATA box sequences are underlined, and the consensus intron-splicing signals, PuCTPuAC (Pu, A or G), are italicized and underlined. Two regions used for PCR primers are indicated by asterisks above the nucleotide, and the putative N-terminal amino acid of the mature protein, serine 71, is indicated by †.

mixed with 2 volumes of 0.8 M NaOH and 1 volume of Folin reagent (1:2 in H_2O; Sigma). The proteinase activity was determined by measuring free tyrosine spectrophotometrically at 660 nm (31). Lamininolytic activity was assayed in the same way as collagenolytic activity except that the final concentration of laminin (Engelbreth-Holm-Swarm mouse sarcoma; Sigma) was 0.1 mg/ml. All of the proteinase assays were done in duplicate at 37°C.

For all inhibition studies, the enzyme was preincubated for 20 min with the inhibitor and all other assay components except the substrate, *N′*,*N′*-dimethyl hemoglobin. After addition of the substrate, the enzyme activity was measured as described above. For the inhibition of the aspartic proteinase by antibodies, the fungal culture medium was preincubated at pH 5.0 for 1 h at room temperature with a mixture of 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM 1,10-phenanthroline, and the indicated amount of antiserum. The residual elastinolytic activity was measured as described above. Protein was measured by the method of Bradford (4).

Preparation and analysis of DNA and RNA. Genomic DNA from *A. fumigatus* was isolated by the method of Kämper et al. (11). Total RNA was isolated by guanidium thiocyanate-sarcosyl extraction (17). Southern and Northern (RNA) blots were prepared as described in standard protocols (34).

PCR amplification of a segment of the aspartic proteinase gene. The genomic DNA of *A. fumigatus* was used as the template to amplify a segment of the aspartic proteinase gene. The following primers were based on two identical segments of two aspergillopepsin genes, *pepO* from *Aspergillus oryzae* (2) and *pepA* from *Aspergillus awamori* (3): sense, 5′CTGGGTCTTCTCCGAGGAG3′; antisense, 5′GAGGGTGGTACCGGTGTCT3′. The amplification protocols consisted of a denaturation step at 95°C for 2 min, followed by 39 cycles of the following steps: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A last elongation step was done at 72°C for 7 min. The resulting 600-bp product was subcloned into pCR^{II} by use of the TA cloning system (Invitrogen, San Diego, Calif.) and sequenced by the chain termination method (35).

Construction and screening of the cDNA library. Poly(A)$^+$ RNA was isolated from a 5-day-old culture of *A. fumigatus* isolate 13 by use of the Fast Track mRNA isolation system (Invitrogen). Double-stranded cDNA was synthesized from 2 μg of poly(A)$^+$ RNA with the RiboClone cDNA synthesis system (Promega Co., Madison, Wis.). After addition of *Eco*RI adapters, the cDNA was ligated with λgt11 arms that were digested with *Eco*RI and packaged in vitro with Gigapack II Gold packaging extract (Stratagene, La Jolla, Calif.), yielding a library of 2×10^6 recombinant phages. Screening of the λgt11 library was performed by plaque hybridization at 42°C overnight with standard reagents (34) containing 50% formamide, with, as the probe, the PCR-amplified gene fragment labeled to a specific activity of $>10^8$ cpm/μg with [α-^{32}P]dATP by the random primer labeling procedure (7). Membranes were washed for 1 h at room temperature in several exchanges of 0.1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) containing 0.1% sodium dodecyl sulfate (SDS) prior to exposure to X-ray films. Positive plaques were identified from the autoradiogram and recovered from agar plates into suspension medium. The insert from the purified positive phage clone (C3) was excised with *Kpn*I-*Sst*I, subcloned into pBluescript KS$^+$ by standard procedures (34), and sequenced by the chain termination method with the Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio).

Construction and screening of the genomic library. Genomic DNA from *A. fumigatus* was partially digested with *Sau*3A and used to generate a genomic library in the λGEM12 vector (Promega). The library was screened by plaque hybridization as described for the cDNA library. Because of the lack of appropriate restriction sites in the positive genomic clone (G30) for subcloning it into a plasmid vector, the open reading frame was amplified by PCR with primers based on the cDNA sequence (C3), subcloned into pCR^{II} vector (Invitrogen), and sequenced. The 5′-upstream and 3′-downstream sequences were obtained directly from the lambda DNA template, with the primers made at about 100 bp inside of the start and stop codon by an automated DNA sequencer (model 373A; Applied Biosystems, Palo Alto, Calif.).

Expression of the aspartic proteinase in *E. coli*. To express both mature and precursor forms of the aspartic proteinase in *E. coli*, the following primers were used: sense primer for the mature protein 5′GGGCATATGAGCTCCGGCA GCGCTGTT3′, sense primer for the precursor 5′GGGCATATGGTCGTCTT TAGCAAA, and antisense primer for both forms 5′GGGCCTAGGTCATC TATGCCTGAGGGC3′. An *Nde*I site in the sense primers and a *Bam*HI site in the antisense primer were introduced for subcloning. The cDNA corresponding to each form of the enzyme was amplified by PCR, the amplified products were cloned into pCR^{II} vector, and the correct insertion was confirmed by sequencing. The *Nde*I-*Bam*HI fragment was excised from pCR^{II} and ligated with pET21b expression vector (Novagen, Madison, Wis.) digested with *Nde*I-*Bam*HI, to generate pET21b-*pepF*. *E. coli* BL21 (DE3) pLysS was transformed with pET21b-*pepF*, and transformants were grown in LB broth containing carbenicillin (100 μg/ml) at 37°C in a shaking incubator. When cells reached the log phase (A_{600} = 0.4), IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 1 mM to induce production of T7 RNA polymerase and thus the synthesis of the aspartic proteinases; cells were harvested after 2 h of growth for further analysis.

Production of polyclonal antibodies. To produce antibodies, the gel segments corresponding to the recombinant protein bands of the mature and precursor forms of the aspartic proteinase were cut out of the SDS-polyacrylamide gel and homogenized in distilled water with a Mini-Bead beater (Bio-Spec Products, Bartlesville, Okla.), and aliquots were injected intradermally into rabbits. After four injections at 2-week intervals, the rabbits were sacrificed and the antisera were collected. The titer of the antibodies was determined by Western blot (immunoblot) analysis.

Electrophoresis and Western blotting. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (15). The gel was electroblotted on Immobilon-P membranes (Millipore, Bedford, Mass.) in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer (pH 11.0) containing 10% methanol. The membrane was blocked with 5% nonfat dried milk in phosphate-buffered saline (pH 7.4), probed with the polyclonal antibodies raised against the aspartic proteinase expressed in *E. coli*, and then monitored by detection with ^{125}I-protein A and autoradiography.

Fractionation of the aspartic proteinase by ion-exchange chromatography. *A. fumigatus* was grown in elastin-containing liquid medium for 6 to 7 days, and the extracellular fluid was concentrated by lyophilization. The lyophilized samples, dissolved in 20 mM bis-Tris propane (pH 6.5), were dialyzed against the buffer overnight at 4°C and applied to a Mono Q column (fast-performance liquid chromatography HR16/10; Pharmacia, Piscataway, N.J.) equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in 20 mM bis-Tris propane (pH 6.5), and fractions were assayed for the aspartic proteinase activity with elastin-Congo red as the substrate. The fractions containing the enzyme activity were dialyzed against distilled water overnight at 4°C, lyophilized, and subjected to SDS-PAGE. The aspartic proteinase was detected by immunoblot analysis.

Immunogold localization of the aspartic proteinase produced by *A. fumigatus* in the lungs of neutropenic mice. Neutropenia was induced in mice by irradiation of the mice, the degree of neutropenia was measured, *A. fumigatus* conidia were introduced intranasally (10^8 conidia per mouse), and lung tissue was sampled at various periods after inoculation as described previously (13). Lung tissue was fixed with paraformaldehyde-glutaraldehyde, dehydrated with ethanol, and embedded in L.R. White as described before (9, 10, 27). Blocks were cut with glass knives on a Richert-Ultracut ultramicrotome. Sections were placed on single-slot grids (2 by 1 mm) coated with Formvar. The coated grids were placed in 5% sodium *m*-periodate for 20 min and then washed five times in distilled water. The grids were transferred into 0.1 M HCl, washed five times in water after 10 min, and immersed for 20 min in 1% bovine serum albumin in 50 mM Tris-buffered saline (pH 7.4) containing 0.1% Tween 20. The grids were placed in 1:100 diluted aspartic proteinase antiserum or control serum for 1 h, rinsed in Tris-buffered saline containing 0.5% Tween 20, and then placed in goat anti-rabbit antiserum conjugated with 20-nm-diameter colloidal gold (Polysciences, Warrington, Pa.) for 30 min before being washed in distilled water. Sections were stained with 2% aqueous uranyl acetate for 15 min, washed in distilled water, and viewed with a Philips 300 electron microscope at 60 kV.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of aspergillopepsin F is L31490.

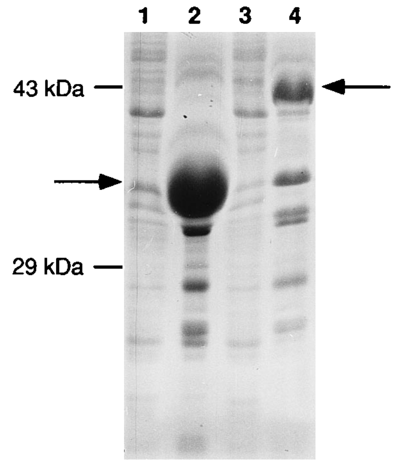

FIG. 5. SDS-PAGE analysis of the precursor and mature forms of *A. fumigatus* aspartic proteinase expressed in *E. coli*. The cell lysate prepared by sonication was centrifuged at 12,000 × *g* for 15 min, and the pellet and supernatant were subjected to SDS-PAGE. The recombinant proteins are indicated by arrows. Lanes: 1, mature-supernatant; 2, mature-insoluble; 3, precursor-supernatant; 4, precursor-insoluble.

RESULTS

Characterization of aspartic proteinase in the culture supernatant of *A. fumigatus*. *A. fumigatus* grown on elastin as a sole nitrogen source has been found to secrete two elastinolytic proteinases into the culture supernatant, an alkaline serine proteinase (13) and a metalloproteinase (19, 38). When these two enzyme activities were inhibited by the presence of PMSF and 1,10-phenanthroline and the activity was measured with ^{3}H-elastin at pH 3.5, about 40% of the total elastinolytic activity manifested at this pH remained; this activity showed a pH optimum of about 5.0 (Fig. 1). This activity is most probably due to an aspartic proteinase. It was not inhibited by pepstatin A (160 μM), DAN (diazoacetyl-norleucine methyl ester; 2 mM), or EPNP [1,2-epoxy-3-(nitrophenoxy propane); 2 mM] (data not shown). The major structural proteins in the lung, i.e., elastin, collagen, and laminin, were all hydrolyzed by the aspartic proteinase. The hydrolysis of these structural proteins by the aspartic proteinase was dependent on enzyme concentration (Fig. 2A to C) and incubation time (Fig. 2D to F).

Cloning and sequencing of the gene and cDNA encoding the aspartic proteinase. To clone the aspartic proteinase gene from *A. fumigatus* by PCR, primers were synthesized on the basis of the homology among the known aspartic proteinase genes (aspergillopepsins) from other *Aspergillus* species. When the genes for two aspergillopepsins, *pepO* and *pepA*, were aligned, two regions of sequences that showed 100% match were found and PCR primers were synthesized on the basis of these sequences. PCR with these primers with *A. fumigatus* genomic DNA as the template yielded a single 600-bp product. The sequence of the PCR product showed a high degree of homology to those of *pepO* and *pepA*. This PCR product was used as the probe to identify the cDNA clone in a cDNA library. Of the four clones thus isolated, the one with the largest insert (C3) showed a size of 1.4 kb. Northern blot revealed a transcript of about 1.4 kb (Fig. 3A). These results suggest that the cloned cDNA represented nearly the full length of the transcript. The cDNA was subcloned into pBluescript KS$^+$ and sequenced. Southern blot analysis showed a single hybridizing band with *Bam*HI and *Hin*dIII and two bands with *Eco*RI-digested genomic DNA (Fig. 3B). Since the gene has an internal *Eco*RI site, the Southern blot results

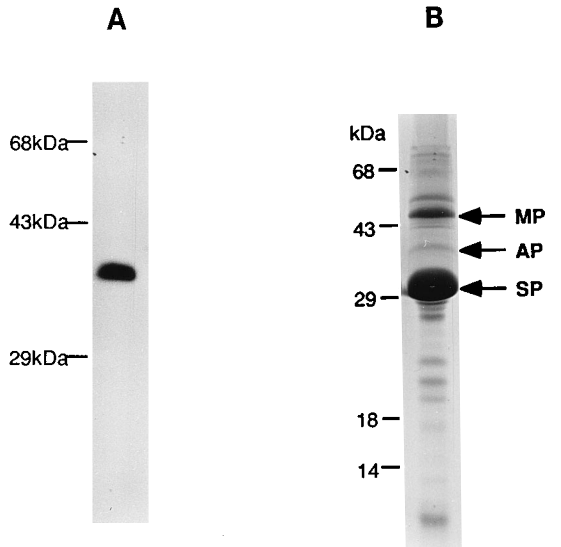

FIG. 6. Immunological detection of the aspartic proteinase in the culture supernatant of *A. fumigatus* grown on elastin. (A) Western blot of the culture supernatant with the antibodies raised against the mature enzyme; (B) SDS-PAGE analysis of the culture supernatant. MP, metalloproteinase; AP, aspartic proteinase; SP, serine proteinase.

indicate that there may be only one aspartic proteinase gene. A genomic library of *A. fumigatus* was screened with the 600-bp PCR product, and one positive clone (G30) was identified and isolated. Because of the lack of appropriate restriction sites for subcloning the DNA fragment from the genomic clone, the entire open reading frame was amplified by PCR from G30 with the primers based on the cDNA sequence. It was then subcloned into pCR^{II} vector and sequenced. The 5′-upstream and 3′-downstream sequences were obtained directly from G30 lambda DNA by an automated DNA sequencer. The nucleotide sequences of the 600-bp PCR product, the cloned cDNA, and the gene all matched perfectly. The cloned aspartic proteinase gene that contains three introns would encode a protein of 393 amino acids (Fig. 4).

Expression of the aspartic proteinase in *E. coli* and antibody production. To express *A. fumigatus* aspartic proteinase in *E. coli*, the segments of cDNA encoding the precursor and the putative mature enzyme were amplified by PCR, and the resulting products were cloned into pET21b expression vector. SDS-PAGE analysis of the lysate of the transformants revealed that IPTG-induced proteinase bands were the obviously dominant ones. The molecular sizes of the expressed proteins were 35 and 43 kDa for the mature and the precursor forms, respectively. To determine whether the expressed proteins were in the soluble form, the insoluble and soluble fractions were separated by centrifugation of the lysate at 12,000 $\times$ *g* for 15 min. SDS-PAGE analysis showed that both the mature and precursor forms were mainly in the insoluble fractions (Fig. 5). Rabbit antibodies were generated against these proteins.

Identification of the aspartic proteinase in the extracellular culture fluid. To identify the protein band that represents the aspartic proteinase in the SDS-PAGE of the extracellular fluid, *A. fumigatus* culture fluid was subjected to ion-exchange chromatography on a Mono Q column. The aspartic proteinase was retained in the column and could be eluted as one peak at 0.4 M NaCl (data not shown). Western blot analysis of the fractions showed that the elution of the enzymatic activity was coincident with the elution of the protein that cross-reacted with the rabbit antibodies prepared against the recombinant aspartic proteinase (data not shown).

Western blot analysis of the unfractionated culture medium showed only one strongly cross-reacting band at 39 kDa, which corresponded to that found in the aspartic proteinase-containing fractions in the ion-exchange chromatography (Fig. 6A). This band corresponded to the protein band that migrated between the alkaline serine proteinase and the metalloproteinase on SDS-PAGE (Fig. 6B). The rabbit antibodies inhibited

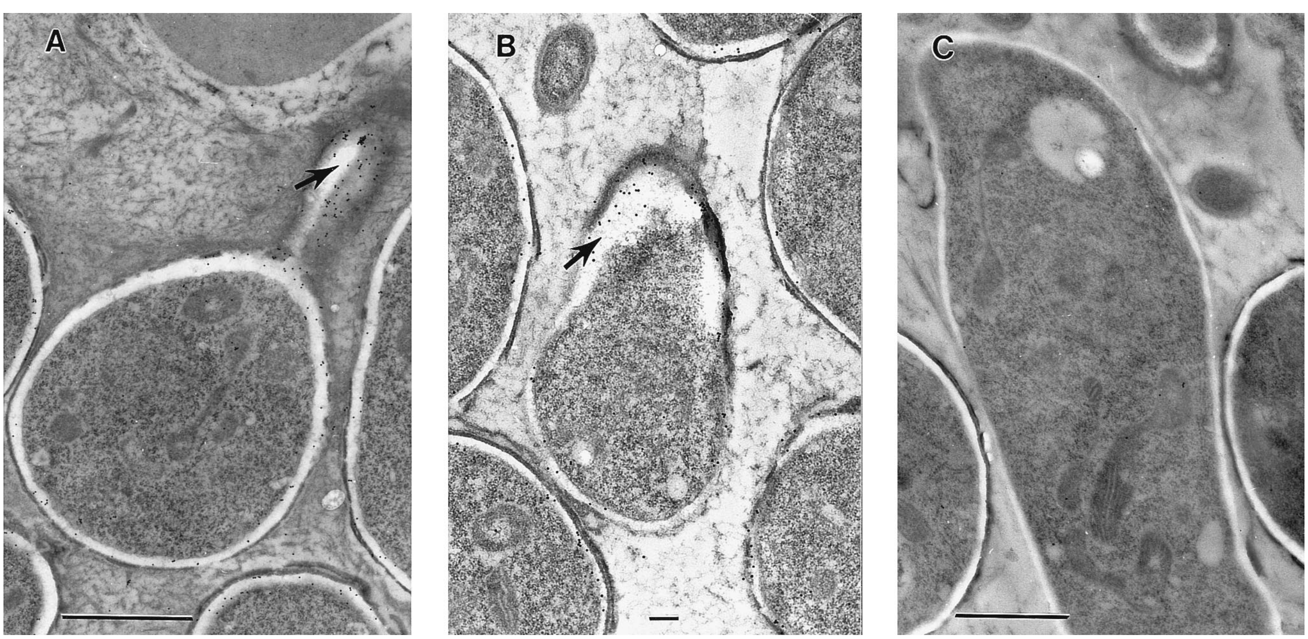

FIG. 7. Immunogold localization of the aspartic proteinase secreted by *A. fumigatus* in the neutropenic murine lung. Tissues were prepared for immunogold labeling 12 h after inoculation with the conidia. Either 100-fold-diluted antiserum (A and B) or 100-fold-diluted preimmune serum (C) was used. The specific gold labelings are densely populated in the walls of germ tubes of the penetrating hyphae indicated by arrows. Bars, 2 μm.

```
         5          15          25          35          45
PepF MVVFSKVTAV VVGLSTIVML -----SLWSS RAKGFTIFLV ARPVTNK--K
PepO MVILSKVAAV AVGLSTVASA LPTGPSHSPH ARRGFTINQI TRQTARVGPK
PepA MVVFSKTAAL VLGLSSAVSA APA------P TRKGFTINQI ARPANKT--R
PepI MVVFSKTAAL VLGLSTAVSA APA------P TRKGFTINQI ARPANKT--R

        55          65          75          85          95
PepF TVNLPAVYAN ALTKYGGTVP DSVKAA--AS SGSAVTTPEQ YDSEYLTPVK
PepO TASFPAIYSR ALAKYGGTVP AHLKSAVASG HGTVVTSPEP NDIEYLTPVN
PepA TINLPGMYAR SLAKFGGTVP QSVKEA--AS KGSAVTTPQN NDEEYLTPVT
PepI TVNLPGLYAR SLAKFGGTVP QSVKEA--AS KGSAVTTPQN NDEEYLTPVT

       105         115         125         135         145
PepF VGGTTLNLDF DTGSADLWVF SSEISASQSS GHAIYKPSAN AQKLNGYTWK
PepO IGGTTLNLDF DTGSADLWVF SEELPKSEQT GHDVYKPSGN ASKIAGASWD
PepA VGKSTLHLDF DTGSADLWVF SDELPSSEQT GHDLYTPSSS ATKLSGYTWD
PepI VGKSTLHLDF DTGSADLWVF SDELPSSEQT GHDLYTPSSS ATKLSGYSWD

       155         165         175         185         195
PepF IQYGDGSSAS GDGYKDTVTV GGVTAQSQAV EAASHISSQF VQDKDNDGLL
PepO ISYGDGSSAS GDVYQDTVTV GGVTAQGQAV EAASKISDQF VQDKNNDGLL
PepA ISYGDGSSAS GDVYRDTVTV GGVTTNKQAV EAASKISSEF VQNTANDGLL
PepI ISYGDGSSAS GDVYRDTVTV GGVTTNKQAV EAASKISSEF VQDTANDGLL

       205         215         225         235         245
PepF GLAFSSINTV SPSD--YFLY TVKSQLDSPL FAVTLKYHAP GTYDFGYIDN
PepO GLAFSSINTV KPKPQTTFFD TVKDQLDAPL FAVTLKYHAP GSYDFGFIDK
PepA GLAFSSINTV QPKAQTTFFD TVKSQLDSPL FAVQLKHDAP GVYDFGYIDD
PepI GLAFSSINTV QPKAQTTFFD TVKSQLDSPL FAVQLKHDAP GVYDFGYIDD

       255         265         275         285         295
PepF SKFQGELTYT DVDSSQGFWM FTADGYGVGN GAPNTNSISG IADTGTTLLL
PepO SKFTGELAYA DVDDSQGFWQ FTADGYSVGK GDAQKAPITG IADTGTTLVM
PepA SKYTGSITYT DADSSQGYWG FSTDGYSIGD GSSSSSGFSA IADTGTTLIL
PepI SKYTGSITYT DADSSQGYWG FSTDGYSIGD GSSSSSGFSA IADTGTTLIL

       305         315         325         335         345
PepF LDDSVVADYY RQVSGAKNSN QYGGYVFPCS TKLPSFTTVI GGYNAVVPGE
PepO LDDEIVDAYY KQVQGAKNDA SAGGYVFPCE TELPEFTVVI GSYNAVIPGK
PepA LDDEIVSAYY EQVSGASGET EAGGYVFSCS TNPPDFTVVI GDYKAVVPGK
PepI LDDEIVSAYY EQVSGAQESY EAGGYVFSCS TDLPDFTVVI GDYKAVVPGK

       355         365         375         385         395
PepF YINYAPSLTQ LYL-LRRHQS NSGLGFSIFG DIFLKSQYVV FDSQGPRLGF
PepO HINYAPLQEG SSTCVGGIQS NSGLGLSILG DVFLKSQYVV FDSQGPRLGF
PepA YINYAPISTG SSTCFGGIQS NSGLGLSILG DVFLKSQYVV FNSEGPKLGF
PepI YINYAPVSTG SSTCYGGIQS NSGLGLSILG DVFLKSQYVV FNSEGPKLGF

       405
PepF ALRHR
PepO AA-QA
PepA AA-QA
PepI AA-QA
```

FIG. 8. Comparison of amino acid sequences of the four aspartic proteinases from *Aspergillus* species, i.e., aspergillopepsins. Identical residues are shaded.

the aspartic proteinase activity at pH 5.0; 40% inhibition was observed with 10 μl of antiserum, and further amounts of antiserum failed to cause more than 50% inhibition (data not shown).

Immunogold localization of the aspartic proteinase secreted by *A. fumigatus* invading the lungs of immunocompromised mice. To test whether the aspartic proteinase is secreted by *A. fumigatus* during the invasion of host lungs, an immunogold electron microscopic localization of the aspartic proteinase was done in the lungs of neutropenic mice inoculated with *A. fumigatus* conidia. Immunogold labeling was done on the lung tissue of neutropenic mice 12 h after inoculation with the fungal conidia. Specific gold labeling was observed when the antiserum prepared against the mature aspartic proteinase was used but not when the preimmune serum of the same rabbit was used (Fig. 7). The gold labeling was found almost exclusively in the cell walls that appear translucent in the electron micrographs, suggesting that the protein was secreted by the fungus in the lungs. The secretion appeared to be targeted towards the germination point, with the dense gold labeling occurring at the germ tube (Fig. 7, arrows).

DISCUSSION

A. fumigatus grown on elastin secretes proteinases into the culture medium. A 33-kDa alkaline serine proteinase (13) and a 43-kDa metalloproteinase (19, 38) have been isolated from the culture medium and characterized. In the present paper,

	96				100					105					110
A. fumigatus	T	L	N	L	D	F	**D**	T	G	S	A	D	L	W	V
A. oryzae	T	L	N	L	D	F	**D**	T	G	S	A	D	L	W	V
A. awamori	T	L	H	L	D	F	**D**	T	G	S	A	D	L	W	V
A. saitoi	T	L	H	L	D	F	**D**	T	G	S	A	D	L	W	V
C. albicans	K	F	N	V	I	V	**D**	T	G	S	S	D	L	W	V
Pepsin	D	F	T	V	I	F	**D**	T	G	S	A	D	L	W	V

			280					285
A. fumigatus	I	A	**D**	T	G	T	T	L
A. oryzae	I	A	**D**	T	G	T	T	L
A. awamori	I	A	**D**	T	G	T	T	L
A. saitoi	I	A	**D**	T	G	T	T	L
C. albicans	L	L	**D**	$\boxed{S}$	G	T	T	L
Pepsin	I	V	**D**	T	G	T	S	L

FIG. 9. Comparison of the conserved regions around the catalytic aspartic residues of the *A. fumigatus* enzyme with other aspartic proteinases. Pepsin, swine pepsin; *C. albicans*, GenBank accession number X56867. The catalytic aspartic residues are in bold, and the conserved DTG (or DSG) motif is boxed. The numbering is based on the aspartic proteinase precursor of *A. fumigatus.*

we report the third elastinolytic proteinase from *A. fumigatus*, an aspartic proteinase. The pH optimum for the enzyme showed that it is an acid proteinase. The activity of this enzyme is not inhibited in the presence of both serine and metalloproteinase inhibitors. It was also not significantly inhibited by compounds such as pepstatin A, DAN, and EPNP, which inhibit many aspartic proteinases. The sensitivity of aspartic proteinases to these inhibitors is known to vary; there are many aspartic proteinases that are not affected by these inhibitors (26), and the *A. fumigatus* enzyme belongs to this class.

All experimental evidence strongly suggests that the aspartic proteinase in the *A. fumigatus* culture fluid is the product of the gene we cloned. The antibodies prepared against the mature protein, expressed in *E. coli*, cross-reacted specifically with a single 39-kDa protein in the fungal culture medium upon immunoblotting. Ion-exchange chromatography of the extracellular fluid showed one aspartic proteinase peak, and the relative activity of the fractions corresponded to the level of the immunologically cross-reacting 39-kDa protein content of the fractions. The antibodies prepared against the recombinant protein inhibited the aspartic proteinase activity in the culture supernatant of the fungus. Thus, there is little doubt that the cloned gene encodes the 39-kDa aspartic proteinase found in the culture fluid. It is apparent from Southern hybridization that *A. fumigatus* contains a single copy of the aspartic proteinase gene.

A. fumigatus aspartic proteinase gene encodes a protein of 393 amino acids and contains three introns. The N-terminal 21 amino acids show the characteristics of a leader peptide. Comparison with other aspergillopepsins indicates that the *A. fumigatus* aspartic proteinase must be synthesized as a precursor containing a 70-amino-acid prepropeptide at the N terminus of the mature protein. The molecular size of the mature protein calculated from the amino acid sequence deduced from the nucleotide sequence is 34 kDa, and it migrates at 39 kDa on SDS-PAGE. Similar anomalous migration has been reported for other fungal proteinases (13, 28), even though the reason for the difference is not clear. We could not detect any carbohydrates on the protein with a glycosylation detection method (GlycoTrack; Oxford GlycoSystems), and no N-linked glycosylation consensus sequence was found in the protein. The amino acid sequence of the precursor form shows 70, 66, and 67% identity to those of *A. oryzae*, *A. awamori*, and *Aspergillus saitoi*, respectively (Fig. 8). The aspartic proteinases of *Aspergillus* species that share the catalytic motif (DTG) with the mammalian pepsin are termed aspergillopepsins, such as aspergillopepsin O (PepO) of *A. oryzae*, aspergillopepsin A (PepA) of *A. awamori*, and aspergillopepsin I (PepI) of *A. saitoi* (36). The present aspartic proteinase of *A. fumigatus* that shares this catalytic motif (Fig. 9) is designated aspergillopepsin F (PepF). That the active-site motif of aspartic proteinases (DTG) and the catalytic aspartic residues are conserved indicate that *A. fumigatus* enzyme belongs to a family of aspartic proteinases.

A. fumigatus aspartic proteinase could be a significant contributor to the virulence of the fungus, with its capability of hydrolyzing the major structural barriers of the lung. Since elastin is a major structural component of lungs, elastin-hydrolyzing enzymes produced by *Aspergillus* species have been considered significant virulence factors in invasive aspergillosis. An alkaline serine proteinase is the major enzyme secreted by *A. fumigatus* grown on elastin, and strong evidence about its contribution to the pathogenicity of the fungus has been presented (13). Although mutants in which the gene for the enzyme was disrupted retained virulence (22, 40), such mutants might produce other proteinases that might compensate for the lack of alkaline proteinase. In fact, a metalloproteinase was found to be produced by such a gene-disrupted mutant (21). An elastinolytic metalloproteinase has been shown to be secreted by *A. fumigatus* penetrating the lungs of immunocompromised mice (19). Aspergillopepsin F, described in this report, is capable of hydrolyzing not only elastin but also collagen and laminin, the other two major matrix proteins of the lung. Even though the level of the aspartic proteinase produced in the elastin-$CaCO_3$ medium is low, much higher levels were found when *A. fumigatus* was grown in casein (18a). Thus, it appears that the opportunistic pathogen uses at least three extracellular proteinases to assist in the invasion through the host's barriers. If such is the case, disruption of a single proteinase gene would not provide a definite answer concerning its role as a virulence factor in invasive aspergillosis.

If the aspartic proteinase detected in the extracellular fluid of *A. fumigatus* culture is relevant to invasive aspergillosis, the fungus invading the host tissue should be secreting this enzyme. To test for such a possibility, the lung tissue of neutropenic mice was examined at different periods after inoculation with conidia of *A. fumigatus*. During the early period when the fungal conidia were germinating and penetrating into the host, immunogold labeling was clearly seen. The fact that the major part of the labeling was found in the cell walls suggested that the aspartic proteinase was being secreted. The localization of the gold particles concentrating specifically at what appeared to be penetrating points suggests that the secretion of the enzyme may be targeted to such sites to aid in the invasion through the host barriers. A similar type of localization of serine proteinase and metalloproteinase was observed previously (13, 19). Thus, the organism might be using a variety of extracellular proteinases, all targeted at the penetration point, to invade the host through the structural barriers.

Aspartic proteinases of the opportunistic pathogen *Candida* species have been found to be the major virulence factors in candidiasis (32). The proteinase-deficient mutants of *Candida albicans* generated by chemical mutagenesis have been shown to be much less virulent than the parent strain, indicating an association between extracellular proteinase and virulence (14). *C. albicans*, the most common etiological agent of candidiasis, is known to secrete multiple aspartic proteinases with broad substrate specificity; they can hydrolyze keratin, collagen, and other matrix proteins (18, 23, 33). The present aspartic proteinase from *A. fumigatus* also showed broad substrate specificity, with ability to hydrolyze elastin, laminin, and collagen, three structural proteins in the host lung. Secretion of *Candida* aspartic proteinases has been demonstrated consistently by immunocytochemistry in tissue specimens obtained from several cases of invasive candidiasis (33).

There are many other examples for the use of extracellular proteinases by infectious organisms in the invasion of host tissues. Protozoan parasites produce different types of proteinases at different stages of the life cycle, and proteinases released by parasites contribute to host tissue damage and parasite invasion (20). Elastases from the opportunistic pathogen *Pseudomonas aeruginosa* have long been considered major virulence factors in the infection by this organism in cystic fibrosis and burn patients (8, 12). Endothiapepsin, an aspartic proteinase produced by the chestnut blight fungus *Cryphonetrica parasitica* (30), and an aspartic proteinase from the plant pathogen *Botrytis cinerea* (24, 25) have also been considered possible virulence factors. Secretion of multiple proteinases that use different catalytic mechanisms could give special evolutionary advantage to the microbes and complicate therapeutic approaches targeted against such enzymes.

ACKNOWLEDGMENTS

This work was supported by grant AI30629 from the National Institutes of Health.

We thank Adam Markaryan for his technical assistance and Debra Gamble for her assistance in preparing the manuscript.

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